Stage-Specific Role of Ets1 Transcription Factor in Natural Killer Cell Maintenance

Undergraduate Honors Research Thesis

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SUMMARY

Natural killer (NK) cells are a group of innate immune cells known for their ability to lyse target cells without prior exposure and are among the first responders in any illness. E26 transformation specific sequence (Ets1) is a developmentally restricted transcription factor found in immune tissues where it is critical for the development of lymphoid cells. Our laboratory and others have previously determined that Ets1 is necessary for complete NK cell development and function by generating an Ets1 global knockout mouse model. However, the Ets1 global knockout mouse model neither assesses the cell extrinsic and intrinsic functions of Ets1 nor does it assess Ets1 dependency at different stages of NK maturation. To address these concerns, we have developed and herein present a novel NK cell specific NKp46^{iCre} mediated conditional deletion of Ets1 in a genetically engineered floxed Ets1 mouse model, NKp46^{iCre}-Ets1$^{fl/fl}$. Using a comprehensive NK cell development panel for multi-color flow cytometry, a 70% reduction of total NK cells was demonstrated in bone marrow, spleen and blood in the NKp46^{iCre} -Ets1$^{fl/fl}$ compared to the Ets1$^{fl/fl}$ and the NKp46^{iCre} controls. We further observed a stage specific reduction in mature NK cells in the NKp46^{iCre} -Ets1$^{fl/fl}$ compared to relevant controls suggesting that Ets1 may be necessary for NK maturation and maintenance in addition to early NK cell development. Our findings not only confirm the intrinsic role of Ets1 in early NK cell development but also introduce a new role for Ets1 in the maintenance of the NK lineage as they mature to cytotoxic NK cells.

INTRODUCTION

1.1 Natural killer cells

Natural killer (NK) cells were first described over 40 years ago as large granular lymphocytes with the unique ability to lyse abnormal cells without prior exposure.[1] Unlike B and T cell antigen receptors, NK cell receptors do not undergo gene rearrangement. Instead, NK cells go through an education process where they gain the ability to recognize the absence or decreased expression of self MHC class I.[1] In addition to their cytotoxic abilities, NK cells have a multitude of roles in the immune system, including the capacity to recruit other innate immune cells through production of TNF, IFNγ and various chemokines.[2] These roles make NK cells an indispensable part of the immune system and led to the large effort that has gone into understanding the intricacies of NK cell development and function.

Development begins in the bone marrow with the generation of NK precursors (NKP) from common lymphoid progenitors (CLP).[3] By definition, NKPs are lineage restricted and will give rise to mature NK cells. These cells express IL2Rβ (CD122) and are negative for markers of other hematopoietic lineages.[4-6] The acquisition of NK cell specific markers, such as NK1.1 and NKp46, begins as NKPs give rise to immature NK cells.[4] Immature NK cells lack the cytotoxic function of mature NK cells but represent an intermediate stage in NK cell development.[4-6] They begin to acquire NK receptors specific for MHC class I molecules in an overlapping manner and go through a process of NK-cell licensing.[5,6] After gaining functional competence in cytotoxicity and production of IFNγ, NK cells leave the bone marrow and take up residency in secondary lymphoid organs or peripheral blood.

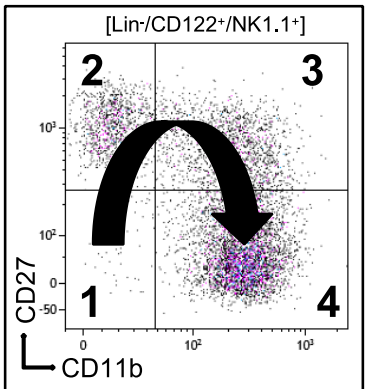

Illustration 1. NK cell developmental stages with CD27 and CD11b.

Most commonly, surface expression of NK1.1 is used to identify NK cells. Subsequently, $CD122^{+}/NK1.1^{+}$ cells can be

divided into a 4-stage process of maturation with surface density of CD27 and CD11b.[7] The progressive upregulation of CD11b followed by downregulation of CD27 defines these stages. Double negative cells (stage 1) represent the least mature subset in this model and maturation continues in a clockwise fashion with the acquisition of CD27 and finally leading to the most mature cells, $CD27^{-}/CD11b^{+}$ (stage 4).[7] Stage 4 in this process represents mature NK cells while the preceding stages are composed of intermediate immature cells. This strategy provides a means to track NK cell development in mice.

1.2 Transcriptional regulation in NK cell development

Lineage commitment from a CLP to a NK cell is determined by a multifaceted network of transcription factors through both repression of alternate-cell factors and promotion NK specific factors.[6] Several transcription factors important in early NK cell commitment cooperate to promote the expression of CD122 as well as to repress B and T cell gene programs. These include inhibitor of E protein transcription factors (Id2), Nfil3, Runx3, EOMES and T-bet.[6,8] Knockout models of the above genes showed normal numbers of CLPs with a reduction in NKP populations.[8] T-bet is essential for IL-15 responsiveness and continues to be important as NK cells mature by modulating expression of a receptor required to egress from the bone marrow.[6,8] Likewise, the importance of Id2 in IL-15 responsiveness has been found by its ability to titrate E-protein activity.[6,8] Both T-bet and Id2 are downstream of another transcription factor critical in early NK development, Ets1.[6, 9,10] Ets1 expression detected prior to the NKP stage and the lack of peripheral NK cells in a global knockout model have revealed its role in early NK cell development. [9, 10] Interestingly, Ets1 expression in human NK cells increases significantly in immature NK cells.[11] This stage specific increase may reflect an additional role for Ets1 in NK cell maturation in later stages.

In many of the above TFs, the mechanisms by which they function are poorly understood. Commonly, only an overall reduction in NK cell numbers has been reported which has made it

difficult to identify when they are important. Recently, conditional knockout models have been used to temporally define the role of many of these factors. In the cases of Tcf1 and Nfil3, when the TFs were conditionally deleted in NKp46^{+} cells, no defects were observed, defining its importance in early NK cell development but not in maturation.[8] This strategy has become increasingly central in defining mechanistic basis of observed phenotypes and continues to uncover more about the complex networks governing NK cell development and maturation.

1.3 E26 transformation specific sequence (Ets1)

Transcription factor and proto-oncogene E26 transformation specific sequence (Ets1) is the founding member of the Ets family of proteins.[12] Ets proteins bind their DNA targets via the winged-helix-turn-helix Ets binding domain composed of 85 amino acids. The most common isoform, p54, has 8 exons and is 97% conserved between mice and humans.[13] In this isoform, the Ets domain is encoded in the last two exons. Targets of Ets proteins are involved in a milieu of cellular processes including proliferation, cell cycle control and differentiation.[14] For this reason, Ets1 overexpression leads to aberrant regulation of these cellular processes and has been implicated in several cancers.[15] Continuing to understand how Ets1 functions is critical in revealing mechanisms of disease.

Although expressed in many tissues during embryonic development, Ets1 expression becomes increasingly restricted to immune tissues as humans and mice age.[13] An Ets1 global knockout murine model was previously used to establish the importance of Ets1 in lymphoid cells.[9, 10, 16, 17] This model was developed by introducing a null mutation into both alleles of the murine Ets1 using homologous recombination.[16] Under this model, T cells displayed proliferative defects and increased spontaneous apoptosis.[16] Ets1 deficient B cells present a phenotype of increased terminally differentiated IgM plasma cells which point to Ets1 importance in B cell differentiation and activation.[17] These data implicate Ets1 as essential for normal B and T cell

maintenance. In contrast, both NK and NKT cells demonstrate a severe reduction in cell numbers in the Ets1 global knockout model.[9, 10, 18] Barton et al (1998) observed a severe reduction in NK cells in the spleen. Ets1 deficient NK cells displayed decreased IFN-γ production along with impaired cytotoxicity to target cells.[9] These results were observed again by Ramirez et al (2012) with an 80-90% reduction of mature NK cells in the bone marrow and spleen of Ets1 deficient mice. From experiments with the global Ets1 knockout murine model, three roles for the transcription factor Ets1 have been described: differentiation of NK cells from hematopoietic stem cells, expression of multiple activating NK receptors (NKRs), and regulation of cytokine response.[10]

MATERIALS & METHODS

Mice and genotyping

Each mouse model was genotyped using PCR with tail snip DNA. The primers, a gift from the laboratory of Dr. Michael Ostrowski, for Ets1 WT (F1), Ets1 floxed (F2), and Ets1 KO (R1) have sequences 5'-CTCTCATTTGCCATC-TTTAGC-3', 5'-GTTTGTTTGTTTGTTTGTTTGTTC-3', 5'-AACACAATATAACATCTTTTT-3' respectively. The primers, a gift from the laboratory of Dr. Jianhua Yu, used to detect NKp46 iCre have sequences 5'-CGAAATTGCCAGAATCAGAG-3' (P1), 5'-CCTTTAGAATGCTTTGCCAA-3' (P2), and 5'-CTTCTGGGATCACACAACCCA-3' (P3). Purified and sorted splenic NK cells were used to verify the deletion of Ets1 by iCre recombinase. NKp46 iCre mice were originally developed as reported in Narni-Mancielli et. al (2011). Mice used for all experiments were between 2 and 5 months old.

Cell Isolation

Spleens and bone marrow were harvested in sterile conditions and placed in phosphate buffered saline (PBS) (cat. #: 14190-144). Single cell suspensions were made by passing cells through 70um filter. Cells were washed and re-suspended in PBS.

Limited cell DNA isolation

Sorted cells were lysed and prepared for PCR using the ARCTURUS® PicoPure® DNA Extraction Kit (Catalog #: KIT0103). In brief, 200 sorted cells were lysed with 12.5uL of PicoPure® DNA Extraction Solution for 3 hours. Standard PCR procedures were used following lysis.

Limited cell RT-PCR analysis of Ets1 expression

Sorted cells were lysed and prepared for cDNA generation using the Ambion Single Cell Lysis Kit (Catalog #: 4458235). Pre-amplification of the cDNA for 14 cycles with the following Taqman primers: GAPDH (ref: mm99999915_G1), NKp46 (mm01337324_G1), Ets1 (mm0117582_mH). The resulting pre-amplified cDNA was diluted 1:5 then subject to 40 cycles of amplification by PCR using primers described previously.

Flow cytometric analysis

Multi-color flow cytometry was used in phenotyping the conditional knockout model. A total of 3e6 cells were stained with fluorochrome labeled antibodies for 30 minutes on ice then washed in PBS. The following antibodies were a generous gift from the laboratory of Dr. Michael Caligiuri: PeCy7-CD27 (LG.7F9/25-0271-82), AF647-NKp46 (29A4.1/137628). The remaining antibodies were purchased from BioLegend (San Diego, CA): CD45-AF700 (clone: 3D-F11/cat. #: 56-045-82), CD19-PE (1D3/553786), CD3-PeCy5.5 (145-2C11/100328), CD122-BV510 (TM-B1/740118), NK1.1-BV421 (PK136/562921), CD11b-FITC (M1-70/562921), Viability-APC-Cy7 (cat. #: L34976). Cells were analyzed on a BD LSRFortessa (Franklin Lakes, NJ) or sorted with BD FACSAria™ Fusion (Franklin Lakes, NJ).

RESULTS

3.1 Development of NKp46^{iCre}-Ets1$^{fl/fl}$ mouse model

To study Ets1 dependence in maturing NK cells, we generated NKp46^{iCre}-Ets1$^{fl/fl}$ mice using a mouse model expressing iCre recombinase under the control of the NKp46 promoter. NKp46 is a NK cell specific natural cytotoxicity receptor involved in forming synapses between NK cells and target cells.[20] NK cells begin to express NKp46 as they transition from immature (i)NK cells to mature (m)NK cells. In this system, iCre recombinase, a site-specific DNA recombinase derived from bacteriophage P1, recognizes locus of X-over P (*loxP*) and catalyzes DNA recombination between *loxP* sites.[21] When employed in a mouse, this can effectively remove a target gene in a tissue specific setting. In the Ets1-floxed (Ets1$^{fl/fl}$) mice (kindly provided by Dr. Michael Ostrowski) *loxP* sites flank exons 7 and 8 of Ets1 (Fig. 1B). The NKp46^{iCre}-Ets1$^{fl/fl}$ model was developed by crossing a NKp46$^{iCre/iCre}$ mouse with an Ets1$^{fl/fl}$ mouse. This resulted in a NKp46$^{iCre/WT}$ (NKp46^{iCre})-Ets1$^{fl/WT}$ mouse which was then crossed with a Ets1$^{fl/fl}$ mouse to get an NKp46^{iCre}-Ets1$^{fl/fl}$ conditional knockout mouse (Fig. 1A). Genotypes were confirmed using PCR (Fig 1C).

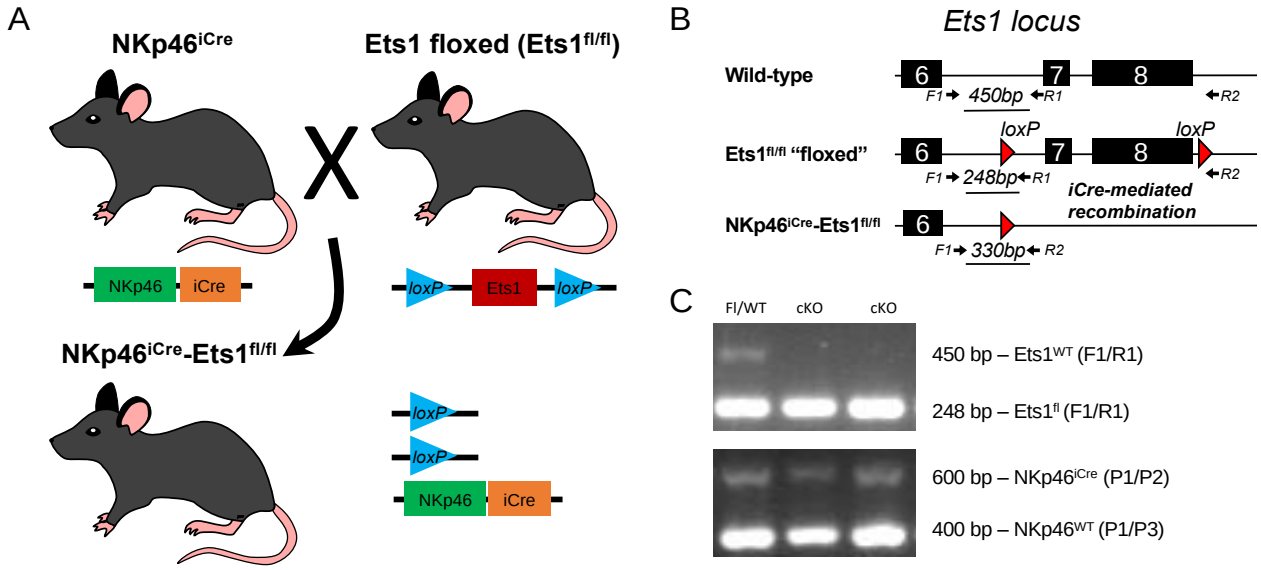

Figure 1. Deletion of the Ets1 gene occurs in NK cells mediated by an NKp46 Cre promoter. (**A**) Breeding scheme to develop NKp46-specific conditional knockout model. (**B**) In Ets1$^{fl/fl}$ mice, Ets-1 exons 7 and 8 are flanked by loxP. When crossed with NKp46 iCre, exons 7 and 8 are deleted via Cre-mediated recombination. **(C)** PCR using tail snip DNA is used to detect the Ets1$^{fl/fl}$ and NKp46 iCre alleles. Lane 1 depicts an Ets1$^{fl/WT}$-NKp46^{iCre} mouse. Lanes 2-3 depict Ets1$^{fl/fl}$-NKp46^{iCre} (conditional knockout) mice.

To verify that iCre-mediated recombination occurred in the crossed mice, genomic DNA was extracted from sorted splenic B ($CD19^+$), T ($CD3^+$), total NK ($NK1.1^+$/$NKp46^+$), and mature NK ($CD11b^+$) cells (Fig 2A). PCR was used to validate that exons 7 and 8 had been removed (Fig 2A). The presence of the 330bp band seen in sorted NK cells and mNK cells occurs only when the exons are removed as a result of primer placement. Concurrently, splenic cells from $Ets1^{fl/fl}$ mice were sorted as a control (Fig 2A). In the $NKp46^{iCre}$-$Ets1^{fl/fl}$ mice, the Ets1 deleted allele was detected most abundantly in mNK ($CD11b^+$) cells compared to the total NK cells, which had some of the wildtype band, and not in the B cells or T cells (Fig. 2A). In the control cells from $Ets1^{fl/fl}$ mice, no Ets1 deleted product was detected and only wildtype product was detected (Fig. 2A).

Additionally, we examined expression of Ets1 transcript in splenic B, T, iNK and mNK cells of $NKp46^{iCre}$-$Ets1^{fl/fl}$ mice to confirm functional iCre-mediated Ets1 deletion. When normalized to the $Ets1^{fl/fl}$ control, mNK cells from $NKp46^{iCre}$-$Ets1^{fl/fl}$ displayed a significant decrease in Ets1 transcript expression (Fig 2B). This decrease was specific to mNK cells and was not seen in iNK cells which had comparable levels of Ets1 transcript to control mice. These results are consistent with the PCR data demonstrating NK cell specific deletion of Ets1 in $NKp46^{iCre}$-$Ets1^{fl/fl}$ mice.

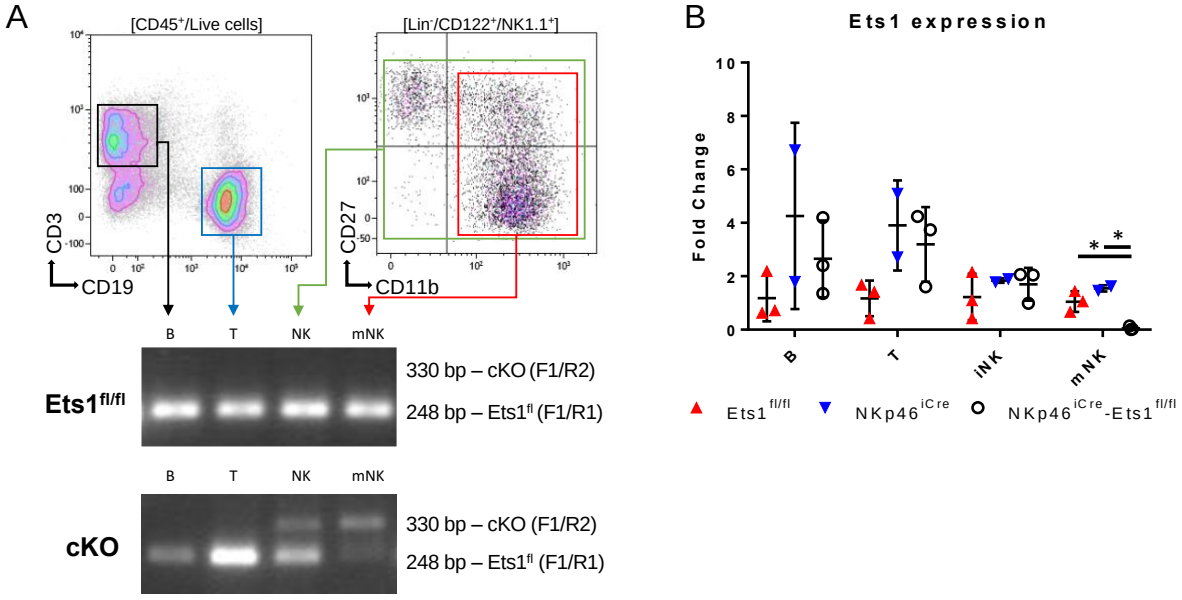

Figure 2. Validation of NK cell-specific Ets1 deletion **(A)** Limited cell PCR from splenic cells of $Ets1^{fl/fl}$ and $NKp46^{iCre}$-$Ets1^{fl/fl}$ (cKO) mice. Sorted B, T, total NK, and mature (m)NK cells show cell specific 330bp conditional knockout band. (n=3) **(B)** 200-cell RT-PCR of Ets1 expression across splenic cells from $Ets1^{fl/fl}$, $NKp46^{iCre}$ and $NKp46^{iCre}$-$Ets1^{fl/fl}$ mice. Immature (i)NK cells are $CD27^{+/-}$/$CD11b^-$). mNK cells show significant decrease in Ets1 expression. (n=2)

3.2 NKp46^{iCre}-Ets1$^{fl/fl}$ mice exhibit reduced CD122^{+} NK1.1^{+} cells

To characterize the effects of NK cell specific Ets1 deletion, lymphocyte populations from spleen, bone marrow and blood were analyzed using multi-color flow cytometry. Total NK cells (CD122^{+}/NK1.1^{+}) displayed a decrease in all compartments compared to control mice (Fig. 3A). These results are consistent with previous reports in an Ets1 global knockout mouse model.[9, 10] B cells and T cells from NKp46^{iCre}-Ets1$^{fl/fl}$ mice were unaffected by the NK specific Ets1 deletion (Fig. 3B). Taken together, these data further validate our model and confirm the intrinsic role of Ets1 in NK cell development.

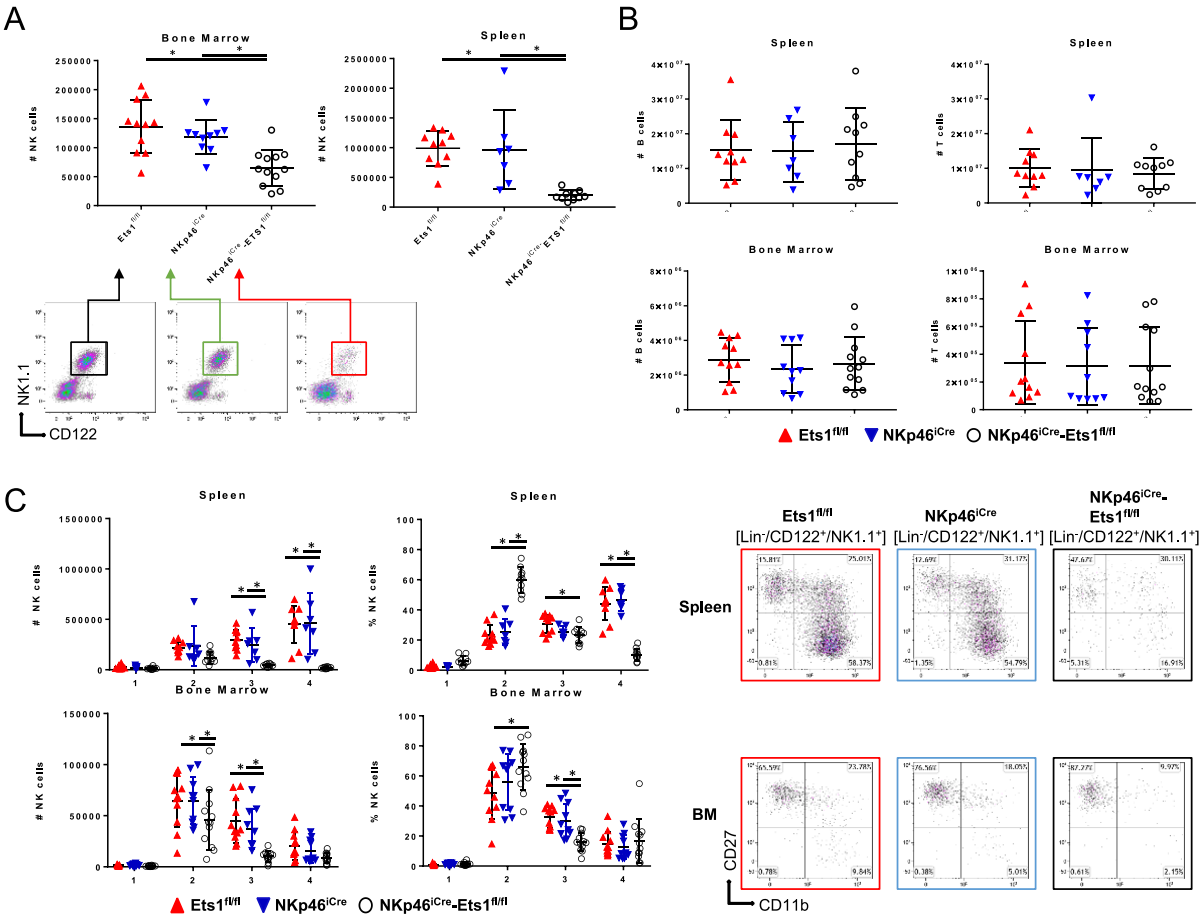

Figure 3. Flow cytometric analysis of lymphocytes in NKp46^{iCre}-Ets1$^{fl/fl}$ **(A)** Total NK cell (CD122^{+}/NK1.1^{+}) in spleen and bone marrow. Below are representative flow diagrams of population from Ets1$^{fl/fl}$, NKp46^{iCre}, and NKp46^{iCre}-Ets1$^{fl/fl}$ mice. **(B)** B and T cells from bone marrow and spleen have normal cell numbers across mouse models. **(C)** NK cell developmental stages using CD27 and CD11b. Representative flow diagrams of at least 10 mice per group. Mice used in these experiments were 2-5 months old.

3.3 Cell intrinsic Ets1 is required for NK cell maturation

To determine the role of Ets1 in NK cell development, we compared the four stages of NK cell development from NKp46^{iCre}-Ets1$^{fl/fl}$, Ets1$^{fl/fl}$, and NKp46^{iCre} mice in both bone marrow and spleen. NK cells from NKp46^{iCre}-Ets1$^{fl/fl}$ mice had an increased proportion of immature cells than those of the control mice. This was characterized by an accumulation of percent CD27^{+} population in all compartments (Fig. 3C). However, total numbers of each stage showed an overall decrease (Fig. 3C). Thus, Ets1 has a stage specific role in the transition of immature to mature NK cells.

DISCUSSION

Herein we introduce a novel conditional knockout mouse model, NKp46^{iCre}-Ets1$^{fl/fl}$, and demonstrate that the Ets1 transcription factor has a stage-specific role in the maturation and maintenance of NK cells. Previous reports have described the role of Ets1 in early NK cell development through its regulation of ID2 and T-BET, cytokine responsiveness, and expression of several activating NK cell receptors.[9, 10] Our report defines a new role for Ets1 in NK cell maturation and maintenance in a cell intrinsic, stage-specific manner.

Townsend et al. (2004) identified T-BET as an important factor for terminal maturation of NK cells in a T-BET knockout mouse model. NK cell numbers in this model were decreased in peripheral organs but increased in the bone marrow reflecting a developmental block in the absence of T-BET.[22] The decrease in overall numbers of NK cells yet increased proportion of immature NK cells in bone marrow, and spleen in NKp46^{iCre}-Ets1$^{fl/fl}$ mice may reflect defects in proliferation, homeostasis or survival. In a global knockout model of Zeb2, a transcription factor important in NK cell maturation, lack of mature NK cells was found to be a product of poor responsiveness to IL-15 and subsequent reduced survival.[23] Interestingly, a similar phenotype was described in T cells of an Ets1 global knockout model.[16] The T cells in this model showed increased spontaneous apoptosis and failure to proliferate when stimulated. This could explain the observed decrease in immature NK cell populations (CD27^{+}/CD11b^{-}) when Ets1 is knocked out, but further experimentation is required to distinguish such a possibility from a defect in proliferation or response to stimuli.

Likely scenarios could be hypothesized based on available data describing the consequence of Ets1 knockout in other models, however, the severe reduction in the size of the cell population under study complicates functional assays that could be performed. To explore these individual avenues, we intend to make use of new techniques, such as mass cytometry, that allow study of limited cell numbers.

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